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The N-linked Glycan g15 within the V3 Loop of the HIV-1 External Glycoprotein gp120 Affects Coreceptor Usage, Cellular Tropism, and Neutralization

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We have studied infectivity and neutralization of X4, R5, and R5X4 tropic HIV-1 mutants, which are lacking N-linked glycosylation sites for glycans g13, g14, g15, and g17 in the V3 loop region of gp120. X4-tropic NL4-3 mutants lacking combinations of g14/15 or g15/17 showed markedly higher infectivity in CXCR4-specific infection. The role of g15 in CCR5-specific infection was investigated using viruses with high (NL-918, R5-monotropic), medium (NL-991, R5-monotropic), and low (NL-952, R5X4-dualtropic) CCR5-specific infectivity. For NL-991, a reduction of infectivity on GHOST-CCR5 cells was observed for a mutant lacking g15. For NL-952 mutants all lacking g15, a complete loss of CCR5-specificity was observed and NL-952 was shifted from R5X4 to X4 tropism. For all mutants of NL4-3, NL-991, and NL-952, where the lack of g15 markedly influenced infectivity or coreceptor usage, neutralization was enhanced. In contrast, NL-918 mutants with or without g15 showed no difference in neutralization and no difference in GHOST-CCR5 infection rates. Thus, for viruses with a low or medium CCR5-specificity the role of g15 for changing CCR5-usage and sensitivity to neutralization was more significant than for viruses with high infection rates on GHOST-CCR5 cells. Our data demonstrate that V3 glycans play an important role in the usage of CXCR4 and CCR5. The lack of g15 was relevant for a more efficient use of CXCR4, whereas interaction with CCR5 was facilitated in the presence of g15. This study also demonstrates that glycan g15 is involved in blocking of neutralizing antibodies and shifting HIV tropism from R5X4 to X4.

Key Words: HIV-1; tropism; V3 loop; glycosylation; coreceptor usage; CCR5; CXCR4.

INTRODUCTION

The human immunodeficiency virus type-1 (HIV-1) external glycoprotein gp120 mediates cell entry by binding specific receptors on the surface of the target cell. These receptors are CD4 (Dalgleish *et al.,* 1984; Klatzmann *et al.,* 1984) and chemokine receptors identified as the HIV-1 coreceptors (Alkhatib *et al.,* 1996; Deng *et al.,* 1996; Feng *et al.,* 1996; Dragic *et al.,* 1996). The chemokine receptors CXCR4 and CCR5 are the two major coreceptors used by HIV-1 isolates (Zhang *et al.,* 1996). All known virus isolates are at least using CXCR4 or CCR5 for viral entry. Viruses using only CXCR4 are called X4 monotropic viruses, whereas viruses using only CCR5 are R5 monotropic. In addition to these monotropic isolates, viruses able to infect cells using CCR5 and CXCR4 are identified, designated R5X4 dualtropic viruses (Collmann *et al.,* 1992; Doranz *et al.,* 1996; Simmons *et al.,* 1996).

In addition to CXCR4 and CCR5, several other chemokine receptors are known to act as cofactors for viral entry (Berger *et al.,* 1998). However, viral infectivity *in vivo* was always identified in correlation to CXCR4 or CCR5 usage, indicating that these two coreceptors play a dominant role for viral infectivity and AIDS pathogenesis (Zhang *et al.,* 1998a,b). Early in infection, R5 monotropic HIV-1 isolates are predominant in patients. During disease progression, viruses can be detected which are using CXCR4 in addition to CCR5 (Smyth *et al.,* 1998) and finally, X4-monotropic viruses are emerging in late stages of infection. The emergence of the X4 isolates in patients correlates with the transition from the asymptomatic to the symptomatic stage of HIV infection and the development of AIDS (Tersmette *et al.,* 1988, 1989; Schuitemaker *et al.,* 1992).

While the mechanism of coreceptor switching from R5 to X4 tropism is still unclear, the interactions between the gp120 and the two coreceptors CXCR4 and CCR5 are better understood. Based on indirect experimental approaches, such as site-directed mutagenesis and infectivity studies using the resulting recombinant viruses, a role of the gp120 V3 loop in determining coreceptor usage was clearly demonstrated (DeJonge *et al.,* 1992; Cocchi *et al.,* 1996).

The V3 loop is one of the five variable regions of the envelope protein gp120, a protein that is extensively glycosylated, containing 24–26 potential glycosylation sites (24 for HIV-1_{NL4-3} strain, 26 for HIV-1_{SF2}) (Zhu *et al.,* 2000). The various glycans present at these sites represent more than 50% of the gp120 molecular mass (Leonard *et al.,* 1990; Lasky *et al.,* 1986). Five of the HIV-1_{NL4-3}

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glycosylation sites, designated g13, g14, g15, g16, and g17, are located in the V3 loop region (see Fig. 1).

Recently we have demonstrated that two of these glycans play a role in CXCR4-specific infection (Polzer *et al.,* 2001). Infectivity and resistance against SDF-1 inhibition of HIV_{NL4-3} mutants lacking glycan g15 was enhanced in contrast to mutants containing fully glycosylated V3 loop. The lack of the two V3 glycans g15 and g17, especially, markedly enhanced infectivity and SDF-1 resistance for CXCR4-expressing cells. To study the function of V3 loop glycans, most site-directed mutagenesis studies have been conducted with X4 tropic HIV-1 strains (Schonning *et al.,* 1996a,b; Nakayama *et al.,* 1998; Losman *et al.,* 1999) and only limited data are available for R5X4 dualtropic viruses (Ogert *et al.,* 2001; Pollakis *et al.,* 2001).

Therefore, we have investigated the role of V3 loop carbohydrates for viral infectivity using a complete set of X4, R5X4, but also R5 monotropic viruses. R5 and R5X4 mutants were constructed by transferring the V3 region of HIV primary isolates into the HIV_{NL4-3} molecular clone. Thus, all mutants had an identical genetic background only differing in the V3 loop region. Based on these X4, R5, and R5X4 viruses, 24 mutants with different combinations of the N-glycosylation sites g13, g14, g15, and g17 in the V3 region were constructed and tested for coreceptor-specific infection and neutralization.

This study presents conclusive evidence that V3 loop glycans are affecting the interaction between gp120 and the coreceptors CCR5 and CXCR4 in a different manner. In CXCR4-specific infection glycans are masking the V3 loop and lack various high mannose (HM) and complex sugars (C), i.e., g14 (HM), g15 (C), and g17 (HM) markedly enhanced CXCR4-dependent infectivity. In CCR5-specific infection the lack of g15 was down-regulating the CCR5-specific infectivity of R5 tropic viruses. Interestingly, the CCR5-specificity of a R5X4 dualtropic virus was completely lost due to the lack of g15. Altogether, our data demonstrate that glycan g15 especially plays an important role in gp120—coreceptor interaction and also in shifting the viral phenotype from R5 to X4 monotropism.

RESULTS

Generation of NL4-3 mutants of X4, R5, and R5X4 phenotype

The NL4-3 wild-type virus is a X4 monotropic virus (X4-NL4-3) presenting a V3 region with five sites for N-linked glycosylation (Fig. 1). To study the role of V3 loop glycans for coreceptor interactions of R5 and R5X4, tropic viruses the V3 region of X4-NL4-3 was replaced by the V3 region of the three primary isolates PI-991, PI-918, and PI-952. The resulting mutants were designated R5-918, R5-991, and R5X4-952. Based on these viruses, we have constructed mutants differing in V3 loop glycosylation (Fig. 2). Five mutants of X4-NL4-3 were constructed to study the role of g14 and g15 for viral infectivity. Twelve R5X4-952 mutants were constructed differing in the presence of glycosylation sites g13, g14, g15, and g17 and three R5-918 and R5-991 mutants of these R5 monotropic viruses were generated differing in g15 and g17. Changes of the glycosylation sites were made by altering the recognition site for N-linked glycosylation as described earlier (Polzer *et al.,* 2001) or according to other reports in the literature (Ogert *et al.,* 2001; Reitter *et al.,* 1998; Nakayama *et al.,* 1998; Losman *et al.,* 1999; Cheng-Mayer *et al.,* 1999). In total 24 NL4-3 mutants were generated, differing in V3 loop amino acid sequence and presence of glycosylation sites to analyze CXCR4- and CCR5-dependent infectivity.

Coreceptor usage, infectivity, and V3 loop glycosylation

The recombinant viruses generated were used to infect GHOST indicator cells expressing CD4 (GHOST parental) and either CXCR4 (GHOST-CXCR4) or CCR5 (GHOST-CCR5). As shown in Table 1, the replacement of the NL4-3 V3 region against the R5 tropic V3 loops of PI-918 and PI-991 results in a NL4-3 mutant only able to infect GHOST-CCR5 cells. Thus, the original R5-tropic phenotype of the primary isolates PI-918 and PI-991 was obtained only by replacing the V3 loop of the NL4-3 wild-type virus against the V3 loop of the primary isolate. This finding indicates that changing the V3 loop was sufficient to change the original NL4-3 tropism from X4 to R5 monotropism. To generate a dualtropic mutant of NL4-3, the V3 loop was replaced by a V3 loop taken from the primary isolate PI-952. Again, the replacement of the V3 region was sufficient to obtain a NL4-3 mutant showing the original phenotype of the primary isolate. The NL4-3 mutant, designated R5X4-952, utilized CXCR4 and CCR5 almost equally and infected GHOST-CXCR4 as well as GHOST-CCR5 cells.

Infection of GHOST indicator cells with mutants differing in V3 glycosylation revealed that the X4-NL4-3 viruses all lacking g15 were exclusively able to replicate in GHOST parental cells (Table 1). These results are in agreement with our earlier studies where g15 and g15/g17 mutants showed higher infectivity. Here we demonstrate that mutants lacking g14 in combination with g15 showed enhanced infectivity, too. Thus, for X4-NL4-3 monotropic glycosylation mutants the lack of g14 or g17 in combination with g15 leads to a markedly enhanced infectivity for CXCR4 and allows infection even in GHOST-parental cells. It is known that GHOST-parental cells exhibit low background expression of CXCR4, corresponding to $<$10% of that observed for GHOST-CXCR4 cells. As an additional control U87-CD4 cells lacking any CXCR4 background expression were used for infection assays. Using the U87-CD4 cells, no infection was ob-

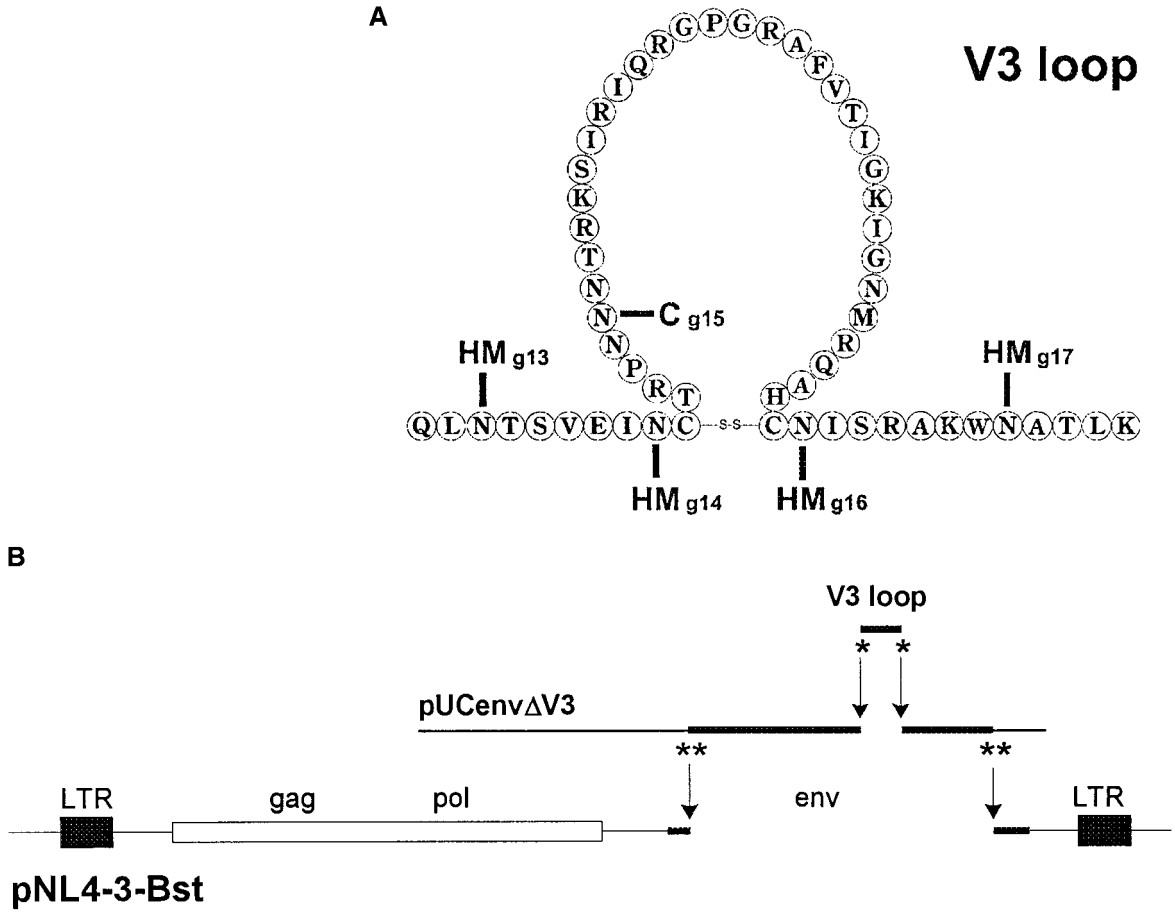

FIG. 1. Replacing the V3 loop region in HIV_{NL4-3} env. (A) The V3 loop region of gp120 contains five recognition sites for N-linked glycosylation. These five sites in the $HIV\text{-}1_{NL4-3}$ gp120 sequence are designated g13, g14, g15, g16, and g17. Glycans g13, g14, g16, and g17 are high mannose (HM) glycans and g15 is a complex (C) glycan. (B) The V3 region was replaced by cloning a V3 fragment flanked by two unique restriction sites (*, *Bgl*II, *Nhe*I) into the env gene present in a standard cloning vector (pUCenvΔV3). V3 fragments were generated by PCR amplification using proviral DNA from primary isolates PI-952, PI-991, and PI-918. Mutants, lacking glycosylation sites, were generated by site-directed mutagenesis using oligonucleotide primers and PCR amplification. After generation of a complete env gene, the env gene was cloned into the retroviral vector pNL4-3-Bst using two unique restriction sites (**, *Bst*EII, *Bam*HI) (Polzer *et al.,* 2001).

served with virus mutants lacking V3 loop glycans (data not shown).

Next we characterized the mutants differing in N-glycosylation containing the V3 loop derived from the primary isolate PI-952. All five mutants containing g15 were able to infect CCR5 as well as CXCR4 cells. A complete loss of CCR5-dependent infectivity was identified in all eight mutants lacking g15 or lacking g15 in addition to other V3 loop glycans. Since the glycosylation sites g13 and g14 were lacking in the original primary isolate PI-952, we have studied three mutants containing the g13/14 sugars (R5X4-952, -g15Q, -g15/17). Presence of g13 and g14 did not facilitate CCR5 usage and infection of GHOST-CCR5 cells was completely negative in the absence of g15 and the presence of g13/14. These data suggest that glycans g13/14 were not involved in gp120–CCR5 interaction. As a control, g15 mutants differing in the first amino acid of the glycosylation site (NNT > QNT) or the third amino acid position (NNT > NNI) were constructed. Again, the lack of g15 was correlated to a complete loss of CCR5 infectivity and no other combination of glycans was able to recover CCR5 usage. Interestingly, the R5X4-952 mutants lacking g13/14/17 and all the mutants lacking g15 showed higher CXCR4-dependent infectivity compared to the mutants containing g15 (R5X4-952, -g14, -g14/17, -g13/14). These results are in agreement with our data on the X4 monotropic mutants, which also showed an enhancing CXCR4-dependent infectivity due to the lack of g15. On the other hand these data show that g15 is crucial for the NL-952 virus to use CCR5.

Since the presence of g15 was important for CCR5 usage by the dualtropic R5X4-952 virus, we characterized mutants of R5 monotropic viruses R5-918 and R5-991. R5 monotropic mutants lacking g15 (R5-918-g15; R5-991-g15) and mutants lacking g15 in combination with g17 (R5-991-g15/17) were tested for CCR5-specific infection. For all three mutants we observed infection of

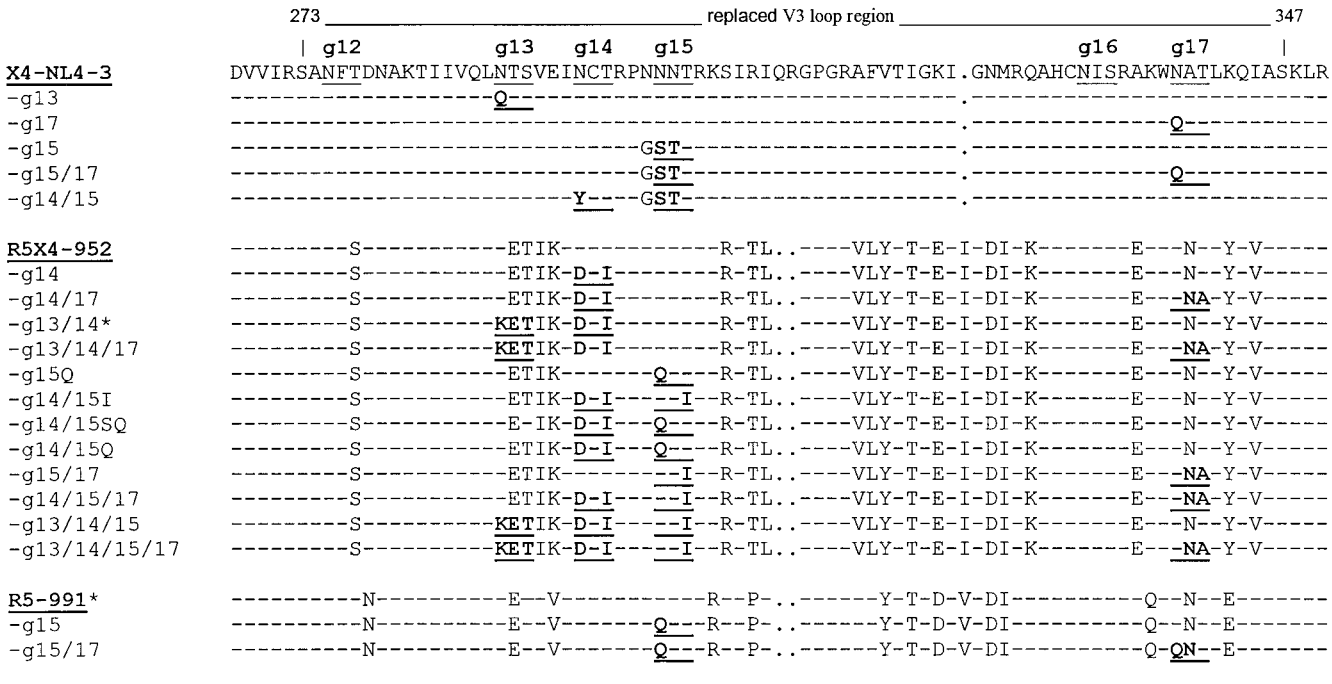

FIG. 2. V3 sequences of X4, R5X4, and R5 tropic HIV_{NL4-3} mutants. The V3 region of HIV_{NL4-3} gp120 from amino acid position 273 to 347 was replaced by the V3 region of a R5X4 dualtropic virus (PI-952) and against the V3 region of two R5 monotropic viruses (PI-918, PI-991). X4-NL4-3, mutants of X4 monotropic HIV_{NL4-3}. R5X4-952, mutants of dualtropic NL4-3 containing the PI-952 V3 loop. R5-918, mutant of HIV_{NL4-3} containing the PI-918 V3 loop. R5-991, mutant of HIV_{NL4-3} containing the PI-991 V3 loop. Bold underlined, mutated recognition sites for N-glycosylation. *, original V3 loop amino acid sequence of the primary isolate.

CCR5-expressing cells. Thus for the R5 monotropic viruses the lack of g15 does not lead to a complete loss of CCR5-specific infection (Table 1). However, CCR5-dependent infectivity was markedly reduced for those R5 mutants lacking g15 (Fig. 3). Thus, for R5 monotropic viruses the lack of g15 was also affecting infectivity in a negative manner. Comparing the CCR5-dependent infection rates of the R5 and R5X4 mutants lacking g15 showed that CCR5-specific infectivity was reduced by 20% for the R5-918-g15 virus, by 50% for the R5-991-g15 virus, and by 100% for the dualtropic R5X4-952-g15Q. Thus, the reduction of CCR5-specific infectivity by g15 was higher for viruses with a lower CCR5-specific infectivity and the reduction in CCR5-specific infectivity was low for viruses with high replication rates on GHOST-CCR5 cells (Fig. 3).

Coreceptor usage, lack of g15, and neutralization

Viruses NL4-3, NL-952, NL-991, and NL-918 with and without g15 were tested for neutralization. As shown in Fig. 4 NL4-3, NL-952, and NL-991 viruses containing the g15 glycan were more resistant to neutralization by a HIV-1 antibody-positive human serum mixture than the viruses lacking g15. As a control, replication of viruses was monitored in the presence of HIV-1 antibody-negative human serum mixture. X4-tropic NL4-3-g15 was neutralized completely compared to the NL4-3+g15 virus. Also CXCR4-specific infection of the NL-952 virus was neutralized more efficiently due to the lack of g15. Neutralization of R5 monotropic NL-991-g15 was also more efficient compared to the neutralization of the NL-991+g15 virus. For NL-918, no difference in neutralization was observed for viruses +g15 or −g15 with normal human serum or HIV-1 neutralizing antibodies.

Taken together, in gp120–CCR5 interaction, the lack of g15 leads to a reduction in CCR5-dependent infectivity. A complete loss of CCR5-dependent infectivity was observed for the dualtropic mutants and a marked reduction in CCR5-usage was observed for the R5 monotropic mutants. Reduction of CCR5-usage due to the lack of g15 was higher in R5-viruses with a low CCR5-dependent infectivity and vice versa. In contrast, viral CXCR4-dependent infectivity was enhanced due to the lack of g15 or to the lack of g15 in combination with g14 or g17. These data suggest that glycan g15 plays an important and different role in gp120–coreceptor interactions due to its enhancing effect on CCR5-specific infection and its inhibitory effect on CXCR4-specific infection. Glycan g15 was also involved in blocking antibody binding to gp120.

TABLE 1

Infection of GHOST Coreceptor Expressing Cell Lines with HIV_{NL4-3} Mutants Differing in X4 and R5 Tropism and V3 Loop Glycosylation

Virus	Presence of V3 loop N-glycosylation sites g13	g14	g15	g16	g17	GHOST cell infection[a] Parental	CXCR4	CCR5
X4-NL4-3	+	+	+	+	+	−	++++	−
-g13	−	+	+	+	+	−	++++	−
-g17	+	+	+	+	−	−	++++	−
-g15	+	+	−	+	+	+	++++	−
-g15/17	+	+	−	+	−	++	++++	−
-g14/15	+	−	−	+	+	++	++++	−
R5X4-952	+	+	+	+	+	−	++	++
-g14	+	−	+	+	+	−	++	++
-g14/17	+	−	+	+	−	−	++	++
-g13/14	−	−	+	+	+	−	++	++
-g13/14/17	−	−	+	+	−	−	+++	++
-g15Q	+	+	−	+	+	−	+++	−
-g14/15I	+	−	−	+	+	−	+++	−
-g14/15SQ	+	−	−	+	+	−	+++	−
-g14/15Q	+	−	−	+	+	−	+++	−
-g15/17	+	+	−	+	−	−	+++	−
-g14/15/17	+	−	−	+	−	−	+++	−
-g13/14/15	−	−	−	+	+	−	+++	−
-g13/14/15/17	−	−	−	+	−	−	+++	−
R5-991	+	+	+	+	+	−	−	+++
-g15	+	+	−	+	+	−	−	++
-g15/17	+	+	−	+	−	−	−	++
R5-918	+	+	+	+	−	−	−	+++
-g15	+	+	−	+	−	−	−	+++

[a] Infection of GHOST cells is expressed as the number of p24 expressing GHOST cells (foci forming units) per 100 ng of viral p24 or per milliliter.

[b] A positive sign (+) indicates: +, 1 to 10 ffu/ml; ++, 10 to 100 ffu/ml; +++, 100 to 10^3 ffu/ml; ++++, $>10^3$ ffu/ml p24 positive cells covering the well. A negative sign (−) indicates that there was no p24 expression detectable in any of the GHOST cells.

DISCUSSION

The insertion of patient-derived env sequences into the HIV_{NL4-3} background is a standard technique used to study coreceptor usage of HIV-1 (Zhang *et al.,* 2001). HIV-1 env is amplified from patient-derived viral RNA or proviral DNA and is inserted into the HIV_{NL4-3} background to generate recombinant viruses that can easily be analyzed (Trouplin *et al.,* 2001; Dittmar *et al.,* 2001). HIV_{NL4-3} was also used to study viral replication and coreceptor usage in response to the presence of V3 loop glycans (Nakayama *et al.,* 1998; Polzer *et al.,* 2001). In general, the HIV_{NL4-3} strain is one of the standard models in HIV-1 research to study gene regulation, infectivity, neutralization, vaccine responses, and *in vitro* pathogenesis.

As demonstrated by these studies, the replacement of the HIV_{NL4-3} V3 region by the V3 loop region of primary isolates was sufficient to change the original X4 phenotype of the HIV_{NL4-3} strain to R5 and R5X4. The coreceptor usage of the R5 and R5X4 primary isolates was conferred to HIV_{NL4-3}. Since the coreceptor usage of recombinant viruses was identical to the coreceptor usage of the respective primary isolate, the present data once again showed that the V3 loop is the most important region of gp120 that determines the affinity of gp120 for the two coreceptors CXCR4 and/or CCR5 (DeJong *et al.,* 1992; Cocchi *et al.,* 1996).

Since the gp120 envelope is highly glycosylated, it was suggested that the glycans are shielding the envelope to prevent binding of neutralizing antibodies. It was shown that glycan g15 especially was protective against neutralizing monoclonal antibodies *in vitro* (Schonning *et al.,* 1996a,b) and *in vivo* (Cheng-Mayer *et al.,* 1999; Malenbaum *et al.,* 2000). Our present data also demonstrated the protective role of g15. In general, g15 can be seen as an immunologically tolerated human structure which is relevant for blocking neutralizing antibodies. Shielding the V3 loop with host derived and therefore immunologically tolerated structures would be a powerful strategy to protect the virus against the strong antiviral immune response directed against the V3 loop. On the other hand shielding the gp120 molecule might hinder HIV-1 in making contact to the coreceptors. Our present data and data from Polzer *et al.* (2001) support this theory for CXCR4-specific infection. For gp120–CXCR4 interactions the lack of g15 or other V3-based glycans leads to higher infectivity, indicating that the sugars next to the V3 loop are blocking gp120–CXCR4 binding. CXCR4-specific infection was also supported for those dualtropic mutants of R5X4-952 lacking g15. The data suggest that the V3 loop is presented in a more open conformation not shielded

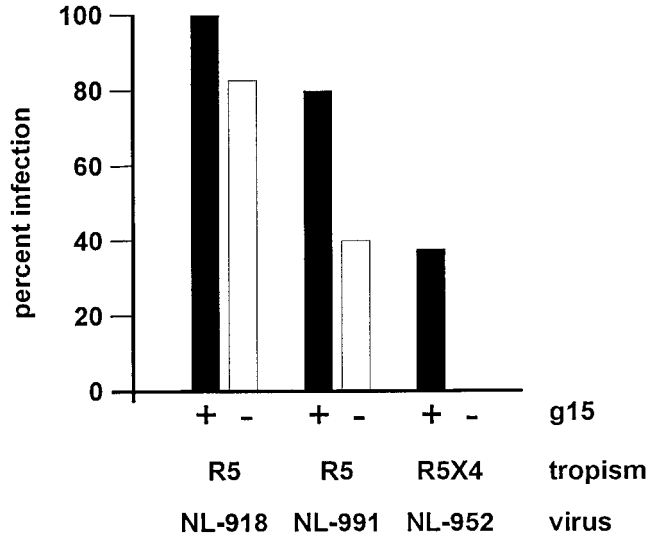

FIG. 3. Lack of g15 and reduction of CCR5-specific infectivity. Infection of GHOST-CCR5 expressing cells was calculated in relation to the mutant with the highest infectivity (infectivity of R5-918 = 100%). +, presence of glycosylation site g15; −, lack of g15. Shown are the averages based on three independent experiments. The standard error of the mean was <10%. Virus inoculums were standardized based on p24 antigen measurements (1 ng p24/10^4 cells/well).

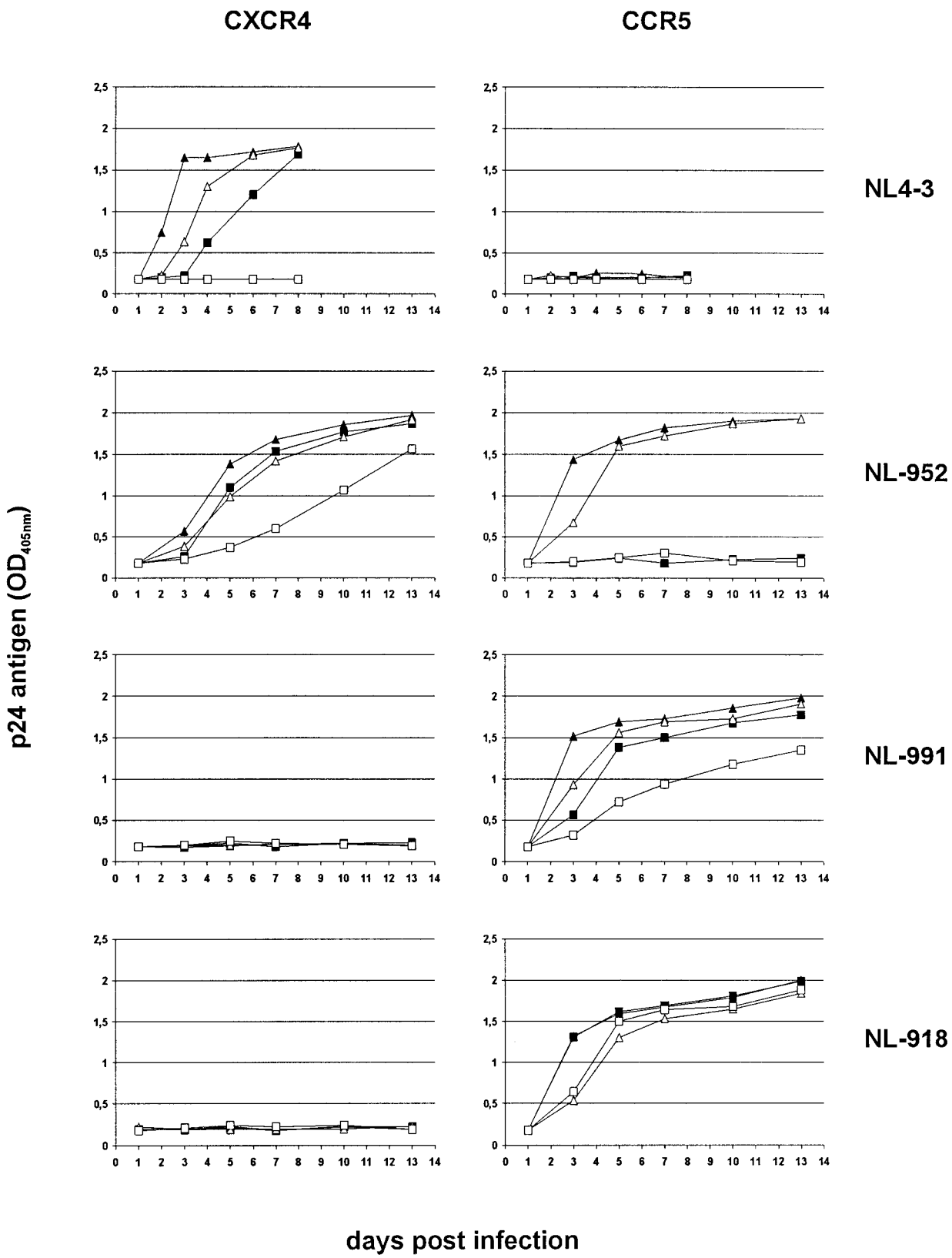

FIG. 4. Neutralization of X4, R5X4, and R5 tropic HIV_{NL4-3} mutants. Viruses differing in coreceptor usage and presence of glycosylation site g15 were tested for replication in GHOST-CXCR4 and GHOST-CCR5 cells (triangles: +g15; squares: −g15). Replication was tested in the presence of normal human serum (black symbols) and a mixture of sera from HIV-infected individuals (white symbols) at a 1:100 dilution. Hela-P4 cells were transfected with the proviral DNA. Cell-culture supernatants were harvested on day 2 and used for infection of U87 cells. Equal amounts of virus were added to each well as calculated by the amount of p24 in the Hela-P4 cell culture supernatant (0.1 ng p24/3 × 10^4 cells for NL4-3 mutants; 0.25 ng p24/3 × 10^4 cells for R5X4-952 mutants; 0.5 ng p24/3 × 10^4 cells for R5-991 and R5-918 mutants). Infection of U87 cells was monitored by a standard p24 ELISA on days 1, 3, 5, 7, 10, and 13 after infection. Shown are the averages of triplicate wells. The standard error of the mean was <15%. Symbols: ▲, +g15 and normal human serum; △, +g15 and anti-HIV serum mix; ■, −g15 and normal human serum; □, −g15 and anti-HIV serum mix.

by the V3-based sugar. That V3 loops of V3-deglycosylated mutants are presented in a more open, more accessible conformation was shown also in the context of antibodies. Antibodies able to neutralize HIV were much more effective against viruses lacking g15 compared to viruses containing the V3 loop glycan (Schonning *et al.,* 1996a; Malenbaum *et al.,* 2000). Thus, for CXCR4-specific infection glycan g15 has an inhibitory function on CXCR4 usage and on antibody binding. On the other hand, lack of g15 promotes higher X4-infectivity but makes the virus highly sensitive to neutralizing antibody.

As stated before, the analysis of the envelope–coreceptor interaction is mainly based on indirect experimental approaches using viral mutants and indicator cell lines. Based on such indirect studies there is a controversy in the literature concerning which region of gp120 is responsible for interaction with the coreceptors. A crystal structure of the gp120 core (Kwong *et al.,* 1998) supports the hypothesis that a conserved region of gp120 is responsible for coreceptor binding. To obtain crystals of gp120 it was necessary to remove the variable loop regions V1/V2 and V3 as well as more than 90% of the carbohydrates normally present on gp120. Another theory proposes that the V3 loop itself binds directly to the coreceptors. This theory is supported by the following facts: (i) HIV mutants lacking the V3 loop are unable to infect cells (Cao *et al.,* 1997); (ii) single amino acid changes promote coreceptor switch from CXCR4 to CCR5 usage (DeJong *et al.,* 1992); (iii) HIV replication can be blocked by V3 peptides (Sakaida *et al.,* 1998; Barbouche *et al.,* 2000); (iv) replacing the V3 loop against the SDF-1 (the natural ligand of CXCR4) amino acid sequence allows CXCR4 usage (Yonezawa *et al.,* 2001); and (v) replacement of the V3 loop against the Mip1-α (the natural ligand of CCR5) sequence allows CCR5 usage (Yonezawa *et al.,* 2001).

This theory is furthermore supported by results obtained from a direct experimental approach for V3 loop–coreceptor interactions (Sakaida *et al.,* 1998). A cyclized V3 loop peptide of HIV strain IIIB (which is identical to the V3 loop of HIV_{NL4-3}) and cyclized V3 loop peptides corresponding to other X4 or R5X4 tropic strains were binding to CXCR4. The V3 loop peptides also inhibited infection by X4-tropic HIV-1 in a dose-dependent manner while other V3 peptides corresponding to a V3 loop of R5 tropic HIV strain ADA had no effect on virus neutralization or CXCR4 binding. Binding to CXCR4 was only observed with cyclized peptides and the linear forms of the V3 loop peptides showed no binding activity for CXCR4 or inhibition of infection. These data indicate that the V3 loop has to be presented in its loop conformation to bind to the coreceptor and that the loop structure is more important for binding than the V3 loop amino acid sequence itself. Using V3 cyclized peptides also suggests that binding of the V3 loop to the coreceptor is independent of other regions of gp120 such as the V1 and V2 loop region or cellular CD4.

In the present study it was shown again that the V3 loop is the most relevant region of gp120 for coreceptor usage and it is highly suggestive that a more open conformation of the V3-loop is relevant for a better use to CXCR4. As can be concluded from our data, the role of glycan g15 for CCR5-specific infection seems to be completely different than for CXCR4. In contrast to CXCR4 glycan g15 is enhancing the binding to CCR5 and therefore seems to be directly involved in V3 loop–CCR5 interaction. In our study all three CCR5-specific NL-viruses showed higher R5-infectivity when the V3 loop was glycosylated and viruses lacking g15 showed reduced infectivity by 20, 50, and 100%. From the present data it is not clear if the g15 effect on CCR5 usage is caused by a direct interaction of g15 with the CCR5 coreceptor or by an interaction of the g15 glycan with the gp120 monomer or trimer on the viral surface. We hypothesize that g15 might interact with a region on gp120 including parts of the V3 loop responsible for, or involved in, CXCR4 binding. The shielding of this region is blocking CXCR4 interaction but has a positive effect on CCR5 usage. If no CCR5-active domain is present on gp120, g15 has no effect on CCR5 usage and only CXCR4 activity is modulated by g15. For dualtropic viruses the shielding of the CXCR4 region might enhance CCR5 usage. However, due to the lack of g15 the CXCR4 region is exposed and in this conformation the R5X4 virus is shifted to the X4 phenotype. If so, the effect of g15 might be directly linked to the overall CCR5-specificity of the respective gp120 or V3 loop. In R5-monotropic viruses the CXCR4-binding region of gp120 might be mutated and less efficient and therefore the shielding effect of g15 might be low or no shielding is necessary anymore to support CCR5 usage. In R5-viruses with no CXCR4-region left no binding of g15 to this region would be possible and therefore the effect of g15 on coreceptor usage would be nondetectable. The binding of the g15 glycan to gp120 might also prevent antibody binding and without the intramolecular interaction gp120 is more accessible to neutralizing antibodies. To test this hypothesis, further work and a much better understanding on the function of neutralizing antibodies against the gp120 envelope complex are needed.

In general, the gp120 and the coreceptor are presented as membrane-bound or membrane-integrated glycoproteins which make it highly difficult to study the protein–protein interaction directly using them in the form of purified proteins. Here, we have used viruses presenting the gp120 in its native conformation and cells expressing both receptors in their native conformation. Based on these experiments we have demonstrated the different role of g15 for CCR5 and CXCR4 interactions. Since we have used identical GHOST cells only differing in the expression of coreceptors, our data also demonstrate that changes in V3 loop glycosylation preferentially

influence the interaction of gp120 with the coreceptors and not with other sugar-binding cellular proteins, i.e., high-mannose binding proteins (Larkin *et al.,* 1989; Ezekowitz *et al.,* 1989), which probably are present on the membrane of the parental GHOST cells as well as the GHOST-CXCR4 or GHOST-CCR5 or U87-CD4 cell lines. Also, infection by X4 viruses lacking g15 can be blocked by SDF-1, the natural ligand of CXCR4. GHOST-CXCR4 and GHOST parental cell infection was shown to be blocked by SDF-1 (Polzer *et al.,* 2001). Thus, the lack of g15 mainly influences the coreceptor-specific interactions and has no influence on CD4 independence. This is supported by the fact that U87 cells, expressing no CXCR4 background activity, were not infected by the -g14/15 mutants in contrast to the GHOST-parental cells, expressing a low CXCR4 background activity.

The binding of the native oligomeric, functional form of gp120 to CXCR4 or CCR5 is more complex than V3 peptide binding to coreceptors and requires conformational changes of gp120 induced as a result of binding to CD4 (Lapham *et al.,* 1996). In context of gp120 or the gp120 trimer, other gp120 regions might influence the binding of the V3 loop to CXCR4. It is conceivable that the so-called conserved coreceptor binding site, as postulated by Kwong *et al.* (1998), is responsible for an intramolecular stabilization of the oligomeric gp120 conformation, holding the V3 loop inside of the molecule to prevent exposure of the V3 loop prior to CD4 binding. The binding of the V3 loop to this intramolecular region might also be influenced by V3-based glycans.

In contrast to the indirect or direct studies on gp120–CXCR4 binding, only limited data are available for gp120–CCR5 binding. In two studies, Ogert *et al.* (2001) and Pollakis *et al.* (2001) demonstrated that g15 played a critical role in coreceptor utilization. Mutants of two viruses, the dualtropic strains HIV-1_{DH12} and HIV-$1_{168.10}$, lost CCR5 function due to the lack of g15. In our present study we also identified the loss of CCR5 utilization by testing V3-deglycosylated mutants of a dualtropic virus. Coreceptor interaction of dualtropic viruses might be more sensitive to V3-deglycosylation since these viruses are specific for both coreceptors, which means they are not fully adapted to only one coreceptor such as the monotropic viruses. Our observation that glycan g15 is necessary for CCR5 interaction is also in agreement with V3 loop sequence data of R5 exclusive viruses (Hung *et al.,* 1999; Trouplin *et al.,* 2001), showing the predominance of g15 in these sequences.

In general, HIV infection and AIDS pathogenesis is characterized by a change of the viral population during progression of the disease. Early in infection CCR5-dependent viruses are predominant and during disease progression the emergence of HIV-1 isolates with the capacity to use CXCR4 (Koot *et al.,* 1993) can be observed. In early infection viruses blocking antibodies by g15 might have an advantage over viruses lacking. In addition to the antibody-blocking function of g15 its enhancing effect on CCR5 binding seems to be a second important step in the battle of HIV against the immune responses in the early stages of HIV-1 infection. The emergence of the CXCR4-specific, X4 tropic strains showing faster replication and higher pathogenicity in patients is associated with disease progression and development of AIDS symptoms. Therefore, it is important to understand how HIV accumulates the capacity to use other coreceptors than CCR5. Our study also shows that R5 monotropic viruses, which are the predominant viruses in the early stages of infection, lost CCR5 affinity in correlation to a deglycosylated V3 loop. This indicates that mutations affecting the N-glycosylation site of R5 viruses might be one important step of the R5 to X4 switch. The data presented do not show why and how the R5 exclusive viruses are gaining the use of CXCR4 to establish the dualtropic phenotype but they do show how such a dualtropic virus can finally switch to the more pathogenic X4 type by a single point mutation.

We hypothesize that a virus containing V3 glycans is protected against antibodies and can bind more efficiently to CCR5 compared to viruses lacking g15. In the early stage of HIV infection this might be an advantage for the virus since the immune response is not impaired. Later in infection, neutralizing antibody responses are lost and viruses can emerge no longer neutralized. In such a situation protection of the coreceptor-binding domain of gp120 by g15 is no longer necessary. As a consequence viruses can emerge lacking V3 glycans such as g15. These viruses can now easily switch to the CXCR4 coreceptor, allowing faster replication, and therefore can grow to higher levels. AIDS progression is the consequence.

In summary, our results demonstrate that the N-linked glycans in the V3 loop strongly influence the R5 to X4 switch. Detection of viruses lacking g15 sugars occurs frequently in the late stage of disease and AIDS patients (Fouchier *et al.,* 1992; Hung *et al.,* 1999; Nelson *et al.,* 2000; Trouplin *et al.,* 2001; Saha *et al.,* 2001), demonstrating the relevance of the glycan g15 in AIDS pathogenesis.

MATERIAL AND METHODS

Construction of NL4-3 mutants with R5X4 and R5 tropism

To generate viral clones differing in coreceptor usage, we have cloned the V3 region of primary isolates in the HIV_{NL4-3} backbone. To isolate the V3 sequences from primary isolates PI-952 (R5X4-dualtropic) and PI-991 (X4-tropic), the proviral DNA from infected human PBMC was amplified by PCR with primers 8693 (5′-CCA ATT CCC ATA CAT TAT TGT GCC) and 8694 (5′-ATA CAT TGC TTT TCC TAC TTC CTG C). Amplified proviral DNA was sequenced to identify V3 sequences flanking the two sites

to generate primers containing the two restriction sites *Bgl*II and *Nhe*I used for further cloning procedures. After amplification using the virus-specific *Bgl*II and *Nhe*I primers (PI-952-BglII-for: 5′-GGT AGT AAT TAG ATC TGC CAA TTT CTC G; PI-991-BglII-for: 5′-GAG ATA GTA ATT AGA TCT GAA AAT T; PI-952-NheI-rev: 5′-CCT CTT AAT TTT GCT AGC TAC CTG; PI-991-NheI-rev: 5′-AAA TTG TTC TCT TAA TTT GCT AGC TAT), the V3 loop region was cloned into the *Bgl*II and *Nhe*I sites, which are unique restriction sites in the envΔV3 derivate (pUCenvΔV3). After V3 cloning, the env gene, containing the V3 sequence of the primary isolate, was inserted into the pNL4-3-Bst recombinant vector by *Bst*EII and *Bam*HI cleavage to generate infectious viral clones as described previously (Polzer *et al.,* 2001).

Generation of NL4-3 mutants differing in the presence of V3 glycosylation sites

The recognition sites for N-linked glycosylation were mutated using site-directed mutagenesis and PCR amplification as described for the X4-NL4-3 mutants (Polzer *et al.,* 2001). For R5X4 tropic viruses a recognition site for N-glycosylation was introduced by mutating the DNA sequence coding for g13 KET to NET or NES. The glycosylation site g14, DCI (originally present in PI-952) was changed to NCT. Site g15 was changed from NNT to NNI or QNT and the glycosylation site g17 was changed from NNT to NNA. For the R5 tropic mutants the glycosylation sites g15 and g17 were mutated by sequence changes NNT to QNT.

Plasmid DNA of pNL4-3 mutants was isolated from *Escherichia coli* bacteria (strain XL-1) by a standard procedure (Macherey & Nagel, Nucleo Spin Miniprep Kit). Purified plasmid DNA was transfected into Hela-P4 cells using the calcium phosphate technique (Chen and Okayama, 1987). Hela-P4 cells were grown as described in the following paragraph to generate viral supernatants. To generate virus supernatants of high titers and virus that contains *in vivo* identical glycosylation, the Hela cell-culture supernatants were transferred to PBMC cell cultures. Virus production was monitored by a standard p24 antigen ELISA (Moore *et al.,* 1990) (Aalto Bio Reagents Ltd., Dublin, U.K.).

Virus cell culture

Hela-P4 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany).

Human peripheral blood mononuclear cells (PBMC) were obtained from HIV-1 negative donors, isolated by Ficoll–Paque gradient centrifugation, and cultivated in RPMI cell-culture medium (PAA Laboratories GmbH, Linz, Austria) containing 10% FCS. PBMC were stimulated by adding 5 μg/ml phytohemagglutinin and 100 U/ml interleukin 2 (Chiron GmbH, Ratingen, Germany). After culturing for 7–10 days, virus production was measured by a standard p24 assay as described above.

Human osteosarcoma (GHOST) indicator cell lines (provided by D. Littman) were cultured in DMEM (Life Technologies), supplemented with 10% FCS. The parental GHOST cell clone (clone 34) expressing human CD4 was grown under G418 selection (500 μg/ml; Life Technologies GmbH). GHOST cells expressing one of the coreceptors CCR5 and CXCR4 were cultivated in medium additionally supplemented with puromycin (1 μg/ml; Sigma, Deisenhofen, Germany).

Evaluation of coreceptor usage

GHOST cells expressing human CD4, CD4+CCR5, and CD4+CXCR4 were used as indicator cells for the characterization of the coreceptor usage of the NL4-3 glycosylation mutants. The monitoring and p24 immunostaining of infected GHOST cells was carried out as described before (Polzer *et al.,* 2001). In brief, GHOST cells were infected with virus supernatants from HIV-infected PBMC cell cultures. Equal amounts of virus were used for infection as measured by the amount of p24 antigen in the supernatants used for each inoculation. Experiments were carried out in triplicate. Infection of GHOST cells was monitored 4 days after infection by a p24 staining using two mouse monoclonal anti-p24 antibodies (EVA365, EVA366, provided by the MRC AIDS reagent project) and a secondary β-galactosidase-conjugated goat anti-mouse monoclonal IgG antibody (Biozol, Germany). Stained blue cell clusters were obtained by staining with x-gal (5-bromo-4-chloro-3-β-D-galactosidase) and counted. Infection of GHOST cells was expressed as foci forming units (ffu) (Chesebro and Wehrly, 1988; Clapham *et al.,* 1992).

Evaluation of viral replication and neutralization

U87 cells (3×10^4 cells/well) expressing human CD4 and either CCR5 or CXCR4 were grown in 96-well cell-culture plates (Greiner, Germany) for 24 h. Cells were infected with equal amounts of virus according to 0.1 ng p24 for infection with NL4-3 mutants, 0.25 ng p24 for infections with R5X4-952 mutants, and 0.5 ng p24 for infections using R5-918 and R5-991 mutants. Prior to infection, virus supernatants from transfected Hela-P4 cells were incubated with HIV-positive or normal human serum at a final dilution of 1:100 for 1 h at 37°C. Cells were inoculated with the virus-serum mixture for 18 h. After inoculation, the cells were washed and cell-culture supernatants were harvested for p24 antigen detection by a standard p24-antigen ELISA on day 1, 3, 5, 7, 10, and 13 after infection. All experiments were carried out in triplicate using fresh supernatants from transfected Hela-P4 cells. Virus was standardized by the amount of p24 that was used for inoculation of GHOST or U87 cells.

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